Chapter 6

INTRACELLULAR GALECTIN-3 FACILITATES TGFβ-INDUCED ENDOTHELIAL-MESENCHYMAL TRANSITION

Byambasuren Vanchin, Ruud Wichers Schreur, Linda A Brouwer, Guido Krenning

Cardiovascular Regenerative Medicine Research Group Department of Pathology and Medical Biology, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands

Manuscript in preparation
ABSTRACT

Endothelial cells give rise to a population of myofibroblasts in cardiac fibrosis through a mechanism called endothelial-mesenchymal transition (EndMT). The process by which EndMT takes place in cardiac fibrosis is incompletely understood. Galectin-3 (Gal-3) has been identified as a novel biomarker for heart failure and its pharmacological inhibition reduced cardiac fibrosis. Here, we investigated if inhibition of Gal-3 is able to attenuate EndMT in vitro. The expression and function of Gal-3 in TGFβ1-induced EndMT was studied in Human Umbilical Vein Endothelial cells (HUVECs). TGFβ1-induced EndMT leads to increased Gal-3 transcription and expression. siRNA-mediated knockdown of Gal-3 attenuates TGFβ1-induced EndMT whereas recombinant GAL-3 did not induce, not aggravate TGFβ-induced mesenchymal transition. Our findings indicate that intracellular Gal-3 facilitates EndMT, suggesting a role for Gal-3 as transcriptional coactivator in EndMT.
INTRODUCTION

Heart failure (HF) is a clinical syndrome in which the heart is unable to pump a sufficient amount of blood to meet the body’s needs. Based on the ejection fraction, the heart failure is classified into the heart failure with preserved, mid-range and reduced ejection fraction (1). Heart failure with preserved ejection fraction (HFrEF) or diastolic dysfunction is more common in older individuals, with risk factors such as hypertension, obesity or coronary artery diseases (2). The pathologic basis of HFrEF is left ventricular remodeling, especially abnormal left ventricular relaxation and an increased left ventricular myocardial stiffness (3). The pathologic basis of the myocardial stiffness in the heart is explained by cardiac fibrosis, which encompasses hyperproliferation of myofibroblasts and excessive deposition of extracellular matrix in the cardiac muscle.

Cardiac fibroblasts are responsible for the production of extracellular matrix (ECM). The ECM serves as a structural scaffold for cardiomyocytes, distributing mechanical forces throughout the cardiac tissue and mediating electronic conduction. The production of ECM is a continuous process where older collagen is broken down and new collagen is deposited (4, 5). However, in response to injuries (e.g. ischemia, hypertension, degeneration etc), fibroblasts undergo a phenotypic transition into myofibroblast (6). Myofibroblasts secrete excessive amounts of collagen and produce alpha smooth muscle actin (αSMA), a protein encoded by the ACTA2 gene. Together with myosin, αSMA forms a contractile complex involved in wound closure (7, 8). The excessive deposition of ECM culminates in several pathologies, such as reduced cardiac contractility, diastolic dysfunction, impaired coronary blood flow and malignant arrhythmias. Altogether, these processes lead to a decrease in tissue compliance and impairs cardiac function, ultimately accelerating the progression of heart failure (9).

The myofibroblasts in cardiac tissue displays a large heterogeneity that can partially be explained by the different origins of the myofibroblasts. Next to resident fibroblasts (10), also bone marrow-derived myofibroblasts (11), circulating monocyte-derived myofibroblasts (12) and endothelium-derived myofibroblasts (13) have been identified. The process by which endothelial cells progressively lose their endothelial functionality and gain myofibroblast-like properties is called endothelial-to-mesenchymal transition (EndMT). A protein associated with heart failure, as well as fibrosis is Galectin-3 (Gal-3), which is encoded by the LGALS3 gene. Increased levels of circulating Gal-3 were measured in heart failure patients (14, 15), and the infusion of Gal-3 into the pericardial sac of rats resulted in increased myocardial fibrosis and cardiac dysfunction(16). Furthermore, the genetic and pharmacological inhibition of Gal-3 in murine models for HFrEF attenuated fibrosis and αSMA expression in fibroblasts compared to the controls(17). Hence, we hypothesized that GAL-3 might be involved in EndMT as an underlying pathological process in cardiac fibrosis. In this study, we investigated effects of Gal-3 on EndMT and identified intracellular Gal3 as a coactivator in TGFβ-induced EndMT.
MATERIALS AND METHODS

CELL CULTURE AND STIMULATION

Human umbilical vein endothelial cells (HUVEC, Lonza, Walkersville, MD) were cultured on 1% gelatin-coated (Sigma, #G9391, St. Louis, MO) flasks or wells. Cells were detached using Trypsin EDTA in PBS (TEP, GIBCO #15400054) solution. Experiments were performed between passage 5 to 7. Cells were cultured in endothelial cell medium (ECM) consisting of RPMI 1640 basal medium (Lonza, #BE12-702F, Verviers, Belgium) with heat inactivated Fetal Bovine Serum (Lonza, 20% v/v), Heparin (LEO, #BE013587 5U/ml), Endothelial Cell Growth Factor (ECGF; own preparation 50 µg/ml), penicillin / streptomycin (Gibco, #15140-122, (1%v/v)) and L-Glutamine (Lonza, #BE17-605E 2mM) at 37°C, 5% CO₂.

Unless stated otherwise, TGFβ1 stimulation was performed by culturing cells for 96h in ECM without ECGF, with 10ng mL⁻¹ TGFβ1 (R&D systems, #240b, batch 15072). Control conditions were cultured in regular ECM. For recombinant human Gal-3 (rh-Gal-3) stimulation, cells were stimulated with TGFβ1 as described, of which the final 18h of stimulation 20µg/mL rh-Gal-3 (Acro Biosystems, GA3-H5129) was added. During TGFβ1 stimulation, medium was refreshed daily.

SIRNA TRANSFECTION

HUVEC were transfected at 60-80% confluency. Prior to transfection, cells were pre-incubated with Opti-MeM (ThermoFischer, #31985062) at 37°C. siRNA transfection mixes (315.6nM siRNA and 315.7 nM Lipofactamine 2000 (Invitrogen, #19155578)) prepared with either siRNA against Gal-3 (LGAL3 silencer select siRNA, Ambion, #4392421) or the control siRNA (All-star negative control siRNA, Qiagen, #1027281). Transfections were performed by adding 26.3 uL Lipofectamine / siRNA transfection mix per cm² culture area. Cells were incubated with transfection mix for 8h at 37°C and 5% CO₂, after which transfection medium was replaced by ECM. After a recovery period of 18h cells were stimulated with TGFβ1 as described earlier.

GENE EXPRESSION ANALYSIS

Cells were lysed in TriZOL (Ambion, #15596-018). RNA was isolated by phenol/chloroform (Emparta) extraction in accordance with the TriZOL manufacturers’ guidelines. RNA was precipitated using 75% Ethanol, air-dried and resuspended in RNase-free water. RNA concentration and purity were determined via Nanodrop 1000 spectrophotometer (Thermo Scientific) and checked the integrity by gel electrophoresis. cDNA was synthesized using the Revert Aid First strand cDNA synthesis kit (Thermo scientific, #K1622) according to the manufacturers guidelines using 500ng RNA as input material. Real-time PCR (ViiA7 Real Time PCR System, Thermo Fischer) was performed by combining cDNA (1ng/µl) with primer master mix (1.2 µm forward and reverse primers, 2x SYBR Green, (Roche, FastStart Universal SYBR Green Master, #04913914001)) in a 1:1 ratio in a total volume of 10µl per well. Data was analyzed using the ViiA7 software (Thermo Scientific) and fold change was calculated using the 2⁻∆∆Ct method. Primer sequences are shown in table 1.
INTRACELLULAR GALECTIN-3 MODULATES ENDOTHELIAL-MESENCHYMAL TRANSITION

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence Forward</th>
<th>Sequence Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>AGCCACATCGCTCAGACAC</td>
<td>GCCCAATACGACCAAATCC</td>
</tr>
<tr>
<td>ACTA2</td>
<td>CTGTTCAGCCATCCTTCAT</td>
<td>TCTGATGCTTGTAGGTG</td>
</tr>
<tr>
<td>COL3A1</td>
<td>CGGAGCCAGGGTCTTC</td>
<td>CACCTGATCCAGTTTCCA</td>
</tr>
<tr>
<td>SNAI1</td>
<td>GCTGCAGACTCTAATCAGA</td>
<td>ATCTCCGAGGTGGGATG</td>
</tr>
<tr>
<td>SNAI2</td>
<td>TGGTTGCTTCCAAGGACAT</td>
<td>GTTGCAGTGAGGGCAAGAA</td>
</tr>
<tr>
<td>TWIST1</td>
<td>AAGGCATCACTATGGACTTTCTCT</td>
<td>GCCAGTTTGATCCAGATT</td>
</tr>
<tr>
<td>S100A4</td>
<td>CGCTTTCTTCTTGGTTTGA</td>
<td>CGAGTACTTGGAAGGTTG</td>
</tr>
</tbody>
</table>

IMMUNOBLOTTING

Cells were lysed in RIPA buffer (Thermo Scientific, #89901) supplemented with protease inhibitor cocktail (Sigma-Aldrich #P8340, 1:100v/v) and phosphatase inhibitor cocktail (Thermo Fisher Scientific, #78420 (1:250 v/v)). Samples were loaded on 4-20% precast gradient gel (Bio-Rad, #4561083). SDS-PAGE gels were blotted onto nitrocellulose membranes (Bio-Rad, #1704270) using the transblot turbo system (Bio-Rad) according to the guidelines of the manufacturer. Membranes were blocked for 1h in odyssey blocking buffer (Li-Cor #92740000). Membranes were incubated overnight at 4°C with primary antibodies for either Gal-3 (Cell Signaling #87985, 1:500v/v) or β-Actin (Cell signaling 4967L, 1:2000). Membranes were washed three times in TBS with 0.1% tween (Sigma, #p2287) (TBST) and incubated with secondary antibody (goat anti-rabbit IgG [Li-Cor #926-68021] 1:10000 v/v in blocking buffer) for 1h at room temperature. Membranes were washed 2 times with TBST, 1 time with TBS and scanned with the Odyssey® CLx (Li-Cor), using the 700 nm channel. Scans were analyzed using Image Studio lite edition (Odyssey, V5.2).

IMMUNOFLUORESCENCE

12 Hours prior to immunofluorescence analysis, transfected and stimulated HUVEC were re-seeded at 80% confluency in NUNC LAB-TEK 8 well chamber slides (Sigma-Aldrich, C7182-1CS). After overnight incubation, cells were fixed with 2% paraformaldehyde for 20 min and permeabilized with 0.5% Triton X in PBS for 5 min. Samples were blocked in 3% Bovine Serum Albumin (Sanquin, #800228065) in PBS for 20 min, and incubated with primary antibodies against Collagen 3 (Cell Signaling, #ab7778 1:50 v/v), or eNOS (BD Pharmingen, #610299 1:1000 v/v) diluted in PBS containing 2% BSA for 1 hour at room temperature. Cells were washed with PBS and incubated with Goat anti-Rabbit IgG (H+L) Alexa Fluor 594 secondary antibody (Invitrogen, #A11037 1:500 v/v) and DAPI (Sigma-Aldrich #D9542 1:5000v/v) in PBS containing 2% BSA for 20 minutes at RT. Image was taken via Leica DM2000 immunofluorescence microscopy.
FLOW CYTOMETRY

Cells were harvested by trypsinization and incubated with CD31-PE (IQ Products, #IQP-552R50 1:10 v/v) and matching isotype controls (IQ Products, #IQP-191 1:20 v/v) in FACS Buffer (2mM EDTA (Sigma, #ED-100G) and 0.5% FBS in PBS) at 4°C for 30 min. Cells were washed in FACS buffer, fixed in 2% PFA (w/v) in PBS for 20 min, washed in FACS buffer, permeabilized in (0.1% Saponin (Sigma, 47036-50G-F), 0.5% FBS in PBS) for 10 min, incubated for 30 min at 4°C with αSMA (Abcam, #Ab7817 (1:50v/v)) and FSP1 (Biorbyt, #ORB88159(1:250 v/v)) conjugated to FITC using the FITC conjugation kit (Abcam, #188285) prior to the staining. Samples were analyzed on a BD FACSCALIBUR flow cytometer. Analysis was performed using Kaluza analysis software (Beckman).

ANGIOGENIC SPROUTING CAPACITY

µ-Slides (Ibidi, #81506) were coated with 10 µl Matrigel (Corning, #354248)) and allowed to solidify for 1 hour. Cells were harvested by trypsinization and seeded at a density of 15,000 cells per well. Images were recorded 24h post-seeding. Sprouting capability was quantified by counting complete hexagonal shapes.

STATISTICAL ANALYSIS

Data are presented as means± s.e.m. Multiple comparison one-way ANOVA was performed to evaluate the difference between groups and p-values < 0.05 were considered to statistically significant.

RESULTS

TGFβ1 INDUCES GAL-3 EXPRESSION IN ENDOTHELIAL CELLS

In order to study the effect of a Gal-3 on EndMT, we induced siRNA mediated knockdown of LGALS3. Silencing of Gal-3 in endothelial cells reduced LGALS3 gene expression to an almost undetectable level (fig. 1a) and a 5-fold (p=0.549) decrease at the protein level was observed. These findings indicate that siRNA mediated intracellular Gal-3 silencing was effective. The TGFβ is main inducer of EndMT, hence we questioned whether TGFβ modulates Gal-3 expression. TGFβ1 stimulation increased the expression of LGALS3 (~3-fold,p<0.05) and protein expression of Gal-3 (p<0.01) compared to unstimulated controls (fig. 1a and fig.1b). These results indicate that TGFβ1 induce Gal-3 gene and protein expression in endothelial cells.
Figure 1. GAL-3 expression is induced by TGFβ1 and successfully inhibited by siGAL3. a) LGALS3 expression levels were determined by quantitative RT-PCR. Under TGFβ1 stimulation, GAL3 is upregulated, whereas siGAL3 transfection successfully decreased GAL3 expression. B) Representative western blots of Gal-3 expression. Quantified GAL-3 protein expression normalized against β-actin. Statistical analysis in this picture: one-way ANOVA test *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001

siGAL-3 INDUCED KNOCKDOWN OF GAL-3 ATTENUATES ENDMT PROGRESSION

Since Gal-3 expression increased upon TGFβ1 stimulation, we studied its effect on EndMT. TGFβ1 induces EndMT in HUVEC via the canonical and non-canonical pathway. HUVECs deficient in GAL-3 had lower expression levels ACTA2 (p<0.05), S100A4 (p<0.01), and COL3A1 (p<0.01), as compared to TGFβ1-treated cells (fig. 2a). At the protein level, αSMA (p<0.0001) and FSP1 (p<0.001) expression was increased in endothelial cells treated with TGFβ1 compared to control HUVECs. Knockdown of GAL-3 reduced the TGFβ1-induced expression of αSMA (p<0.01), FSP1 (p<0.05) (fig. 2b) and Collagen III (fig. 2c).

EndMT is not only characterized by the increased mesenchymal gene expression but also by the loss of endothelial specific gene expression and functions. eNOS expression was higher in Gal-3 deficient cells compared to their control HUVECs. TGFβ1-treatment abrogated eNOS expression and siRNA against Gal-3 mildly rescued the TGFβ1-induced eNOS decline. (fig. 2d)

Moreover, TGFβ1-stimulation lead to less angiogenic sprouting capacity of endothelial cells compared to unstimulated controls. A knockdown of Gal-3 slightly improved the angiogenic sprouting capability after 24h (fig. 2e, fig. 2f). Combined, these data indicate that Gal-3 inhibition attenuates EndMT.
Figure 2. siGAL3 mediated knockdown of Gal-3 attenuates EndMT progression. a) ACTA2, S100A4 and COL3A1 transcription levels were determined by quantitative RT-PCR and normalized against its control conditions. siGAL3 attenuates TGFβ1 induced ACTA2, S100A4 and COL3A1 transcriptional increment. b) Gal-3 deficient endothelial cells have significantly lower expression of αSMA and FSP1 upon TGFβ1 stimulation compared to TGFβ1 stimulated negative controls. c) Collagen III level was evaluated by the immunofluorescence staining and DAPI (blue) and COL3A1 (red) are shown. Upon TGFβ1 stimulation Collagen III protein expression increases, whereas this increment is attenuated by the siRNA against GAL-3. d) Representative immunofluorescence images of DAPI (blue) and eNOS (red). TGFβ1 stimulation reduces eNOS expression, whereas a knockdown of Gal-3 directly increases eNOS expression compared to ECM control conditions. e) Representative bright field images of sprouting capability.
INTRACELLULAR GALACTIN-3 MODULATES ENDOTHELIAL-MESENCHYMAL TRANSITION

ties of HUVECs. Although it is not statistically significant, the TGFβ1 treated cells lose their endothelial functionality compared to their controls. There has tendency that siGAL3 attenuates the loss of endothelial functionality. f) Quantification of angiogenic sprouting capability by scoring intact meshes after 24h. Statistical analysis in this figure: one-way ANOVA test *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001

EXTRACELLULAR GAL-3 DOES NOT MODULATE TGFβ1-INDUCED ENDMT

Galectin-3 facilitates EndMT, albeit through an unknown mechanism. We investigated if Gal-3 signaling through its receptors might induce or aggravate of EndMT by treating HUVEC with recombinant Gal3. rhGAL3 did not induce the expression of αSMA and FSP1 in control endothelial cells, nor did the addition of rhGal3 aggravate the TGFβ1-induced expression of αSMA and FSP1. These findings suggest that extracellular GAL3 does not influence EndMT, but rather intracellular levels of GAL3 modulate EndMT in vitro (fig. 3).

Figure 3. Extracellular Gal-3 doesn’t modulate EndMT. Population of cells undergoing EndMT were analyzed via flow cytometry by double staining for endothelial marker CD31 and mesenchymal marker αSMA or FSP1. TGFβ1-induced αSMA and FSP1 expressions is unaltered by rh-Gal-3. *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001

ENDMT TRANSCRIPTION FACTOR SNAI1, SNAI2 AND TWIST1 ARE MODULATED VIA INTRACELLULAR GAL-3

GAL3 knockdown had no direct effect on the expression of the EndMT transcription factors Sna1, Sna2 and Twist1, whereas TGFβ1 stimulation increased their expression. TGFβ1-stimulated endothelial cells that were deficient in GAL3 had reduced expression levels of SNAI1 (p<0.01), SNAI2 (p<0.05) and TWIST1 (p=0.512), indicating that GAL3 is involved in the transmission of TGFβ1-induced signaling. (fig. 4) These findings suggest that intracellular Gal-3 might be a transcriptional coactivator to TGFβ during EndMT.
DISCUSSION

In this study we found that Gal-3 facilitates TGFβ-induced EndMT. In the absence of TGFβ, Gal-3 does not induce or aggravate EndMT, however in the presence of TGFβ the loss of GAL3 expression inhibits EndMT. In contrast, rhGAL3 didn’t show any effect on both control and TGFβ - stimulated condition, suggesting that this modulation is happening through intracellular Gal-3. Moreover, our data suggests the GAL3 is not an independent modifier of EndMT, but rather acts as an intracellular modulator of TGFβ signaling.

In this study, gene expression and immunoblotting analysis clearly showed that Gal-3 expression was induced by TGFβ1. We also showed that a knockdown of Gal-3 is able to attenuate the expression of EndMT-related transcription factors SNAI1, SNAI2 and TWIST1. However, it remains elusive how GAL-3 modulates this gene expressions. Transcriptional control of fibrosis encompasses a highly interactive multi-protein signaling system including TGFβ, WNT, YAP/TAZ and other signaling mechanism (18). (19). Herein, Gal-3 can bind to the β-catenin/Tcf complex and facilitate its nuclear translocation (20). β-catenin is an important part of the WNT signaling cascade and this finding may explain the merge between TGFβ and WNT signaling in cardiac fibrosis. During idiopathic pulmonary fibrosis, Gal-3 inhibition prevented TGFβ and bleomycin-induced fibrosis via β-catenin phosphorylation and nuclear translocation(21). Combined, these data suggest that during EndMT, GAL3 might act as a transcriptional coactivator for TGFβ signaling. Further research will be needed to identify its signaling partners.

In conclusion, we show here that intracellular Gal-3 facilitates TGFβ-induced endothelial-mesenchymal transition in endothelial cells, possibly via the potentiation of TGFβ signaling.
REFERENCES


